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Antibacterial Effects of the Fluid in *Nepenthes* Pitcher Plant

Abstract

In this inquiry, an analysis of the antibacterial effects of pitcher plant fluid from genus *Nepenthes* was conducted. If pitcher plant fluid is a viable antibiotic, this could potentially be groundbreaking as a new type of disinfectant that is completely natural, and it may even be useful as an antibiotic in vivo. *Nepenthes* pitcher plants contain several enzymes in the bottom of their pitchers, and three of which were identified as having antibiotic effects, according to a proteomic analysis. For our inquiry, pitcher plants were cultivated for several weeks, allowing to accumulate enough fluid. Stock cultures of three different types of bacteria were inoculated: *Escherichia coli, Staphylococcus epidermidis,* and *Streptomyces griseus.* These three species were chosen for their pathogenic properties. Additionally, *S. griseus* is routinely used to produced antibiotics, indicating that it may be resistant to certain antibacterial agents. A zone of inhibition study was conducted using the pitcher plant fluid along with water as a control. The fluid we collected from the pitcher plants showed extensive antimicrobial effect. A 1.3-1.5 cm zone of inhibition around each filter paper disc soaked in pitcher plant fluid presented in *E. coli* and *S. epidermidis* and no colonies of *S. griseus* grew within proximity of the filter paper discs soaked in pitcher plant fluid. To grant statistical significance to our results, three ANOVA tests were conducted with each set of raw data obtained from each plate. P-values of < 0.00001, <0.00001, and 0.1589 were obtained for each type of bacteria, respectively.

Introduction

The pitcher plant, a carnivorous plant of the genus *Nepenthes*, is a plant that has evolved from an exclusively autotrophic diet to a heterotrophic diet (Fukushima & Fang 2008). *Nepenthes* contains highly specialized leaves that have adapted to "trap" insects and arthropods in a pitcher-like system, with fluid at the bottom (Moran 1996). Several mechanisms allow for the efficient capturing and digestion of insects/arthropods, including: pheromones secreted by the plant to attract insects, a slippery rim around the edge of the pitcher itself (so as to cause insects to fall in), and

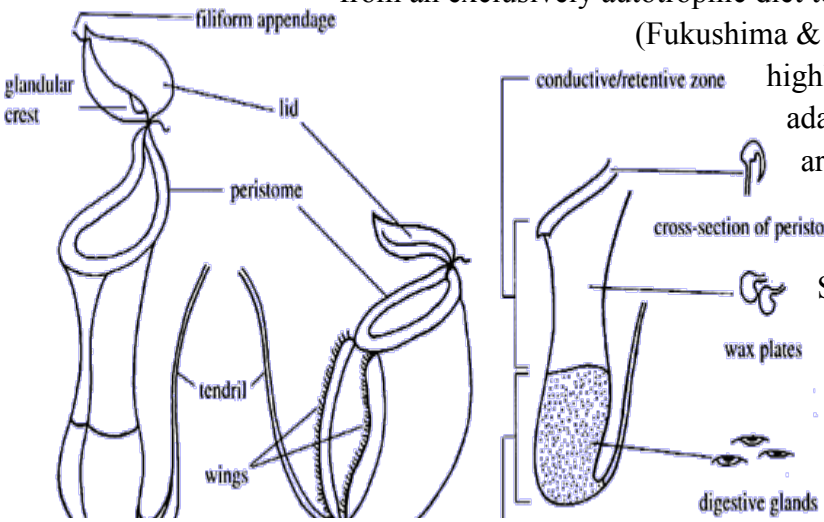


Figure 1: Nepenthes anatomy. [Diagram]. Retrieved from http://sp13ethnobotany.providence.wikispaces.net/CORALINE+-+Nepenthes+raf%EF%AC%82esiana.

digestive enzymes in the pitcher plant fluid at the bottom of the pitcher (Fukushima & Fang 2008). Because the pitcher plant has evolved and adapted these mechanisms, and it is very expensive in terms of resources for the plant to manufacture and maintain the necessary components to keep these features functional, the pitcher and the fluid in the bottom have become the pitcher plants' primary source of nutrients. Though the pitcher plant does contain plant cells (with chlorophyll), these plan cells within the pitcher wall and its leaves are only secondary sources of nutrients, and the plant will never rely solely on these autotrophic mechanisms in order to survive (Schaal & Schwaegerle 1979). One paper published in the *Journal of Proteome Analysis* conducts a full proteome assay of the enzyme composition of the Asian Red Pitcher Plant, a subgenus of *Nepenthes* (Hatano & Hamada 2008). Not only were enzymes identified through proteome analysis, but sequencing using PCR and gel electrophoresis was conducted to also identify the genes responsible for producing said enzymes. Only a mere three out of the seven enzymes present were characterized as having digestive effects. Two enzymes were posited as having some relationship with the pitcher lid opening. And finally, of particular interest, two more enzymes were identified as having antibacterial effects (Hatano 2008). These two antibacterial enzymes were hypothesized to be protective for the pitcher plant and to ensure that all nutrients from insects were received by the plant, not by bacteria living within the fluid. Keeping the pitchers' fluid microbe-free is of utmost importance for *Nepenthes*. Secondly, given that modern antibiotics are becoming highly sophisticated, and new antibiotics are always in high demand thanks to bacterial resistance to antibiotics (CDC 2017), we posit that these enzymes identified by proteome analysis could be extracted from pitcher plant fluid and used as antibiotics, either *in vitro* or even *in vivo*. Our inquiry investigates if these enzymes are at all effective against three common strains of bacteria that are pathogenic or are closely related to pathogenic strains.

Materials and Methods

We began with three pitcher plants of genus *Nepenthes*. Each pitcher plant was grown in BME 2.510 for several weeks to collect enough pitcher plant fluid to use for our inquiry. Each plant was grown under an incandescent or LED light and watered with about 20 mL of distilled water once every two days. When sufficient fluid was detected in the pitchers, a sterile pipet was used to extract about 10-20mL of liquid from each pitcher. This fluid was put into a falcon tube, covered in foil, and stored in the fridge.

During the above three steps, stock plates and tubes of bacteria were made. We obtained 200 µl cultures, each containing a different type of bacteria: *Escherichia coli*, *Streptomyces griseus*, *Staphylococcus epidermidis*. Three stock plates containing *E.* coli, *S.* epidermidis, and *S. griseus* were prepared. Using a sterile inoculating loop for each type of bacteria, we streaked three stock plates (one with each culture) containing LB medium, inverted the plate, and grew them at an appropriate temperature (37 degrees C) until visible colonies formed. Each stock plate was grown for approximately 24 hours. We covered the edges of each plate with parafilm and stored them in the fridge after they had shown sufficient growth. Three stock tubes were also

created. Using a sterile inoculating loop for each type of bacteria, we inoculated three 5 mL tubes containing LB medium with 100 µl of liquid bacterial culture from stock cultures prepared earlier, one with each type of bacteria. All three tubes were grown until they were turbid.

For the zone of inhibition experiment, several filter paper discs were cut using a hole puncher. The discs were autoclaved. Using the stock tubes made several days earlier, one plate was inoculated with each type of bacteria from the liquid stock cultures, making a total of three plates. 100 µl of liquid bacterial culture was pipetted onto each plate and spread. For each plate, four filter paper discs were soaked (two in the pitcher plant fluid, two in distilled water as a control). Two filter paper discs, one of each type, were placed onto the newly inoculated plates several centimeters apart. The Petri dishes sat upright for 20 minutes so that the filter discs adhered to the surface. The plates were then inverted and incubated. After a long incubation period of 48 hours at a temperature of 37 degrees C, the plates were retrieved from the incubator and pictures of the plates were taken. ImageJ was used to compute accurate measurements of the inhibition radius around each filter paper disc.

Results

Qualitative data in the form of pictures from our first (and unsuccessful) trial are shown below (*Figure 2*). These show each type of bacteria and their zones of inhibition around each filter paper disc.

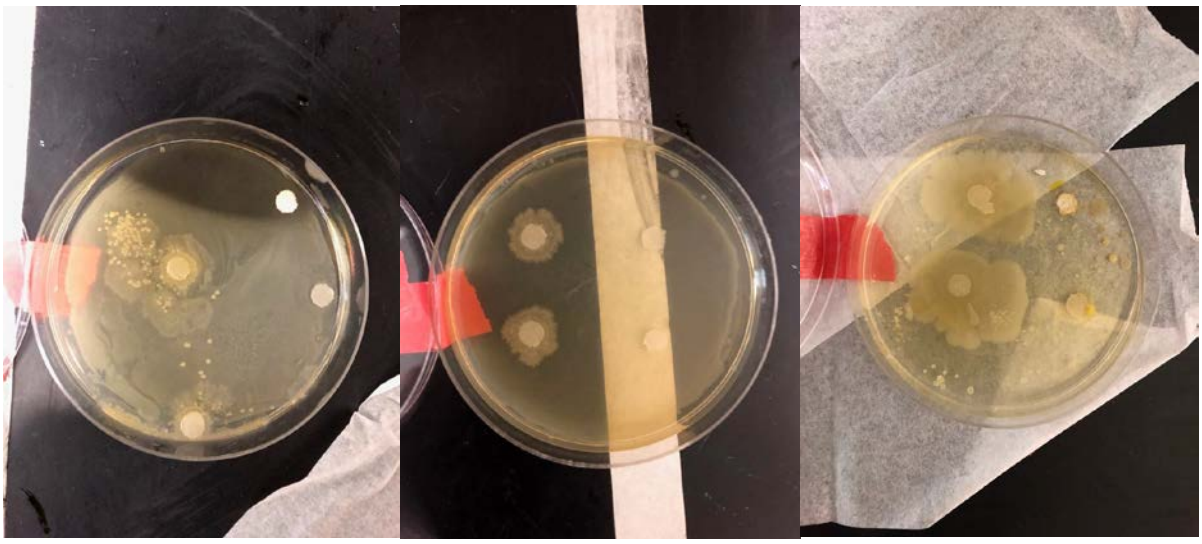


Figure 2. From left to right: E. coli, S. epidermidis, and S. griseus. Filter paper discs soaked in PP fluid are on the left on each plate, and discs soaked in distilled water are on the right of each plate.

Qualitative data in the form of pictures from our second (and successful) trial are shown below (*Figure 3*). These show each type of bacteria and their zones of inhibition around each filter paper disc.

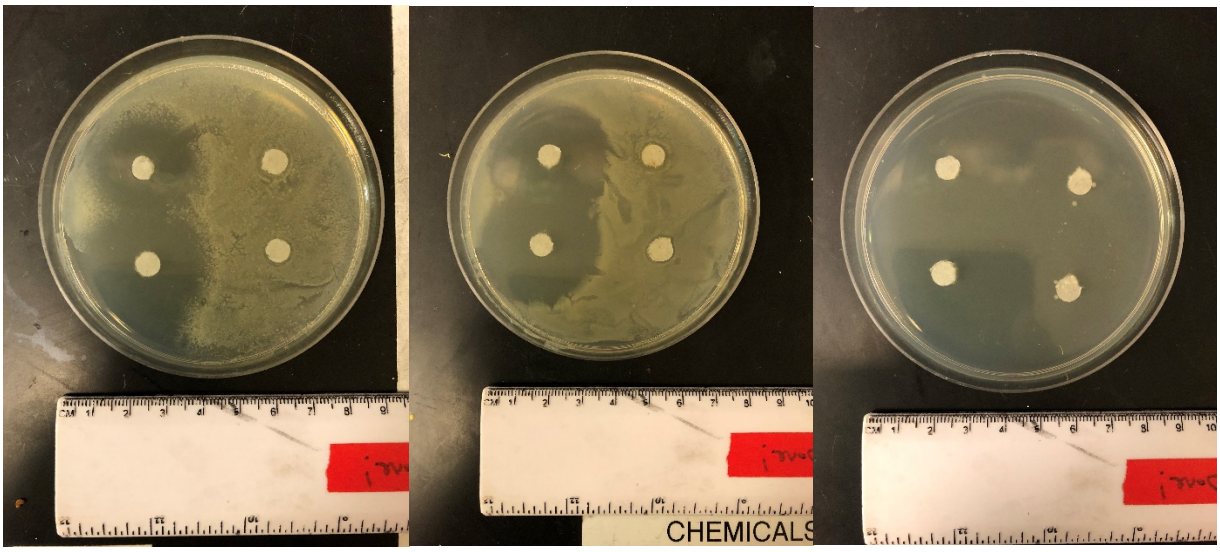


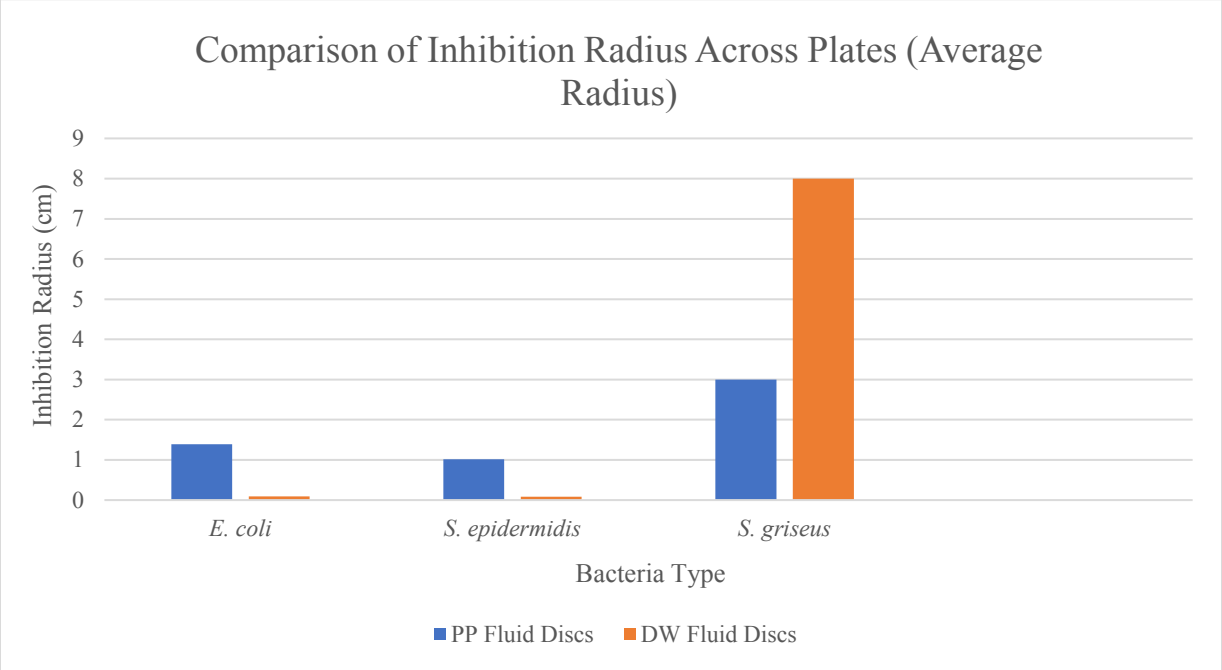
Figure 3. From left to right: E. coli, S. epidermidis, and S. griseus. Filter paper discs soaked in PP fluid are on the left on each plate, and discs soaked in distilled water are on the right of each plate.

For *E. coli* and *S.* epidermidis, ImageJ was used to digitally measure the length from the edge of each filter paper disc to the edge of the zone of inhibition (in centimeters) in three different places for each disc, totaling six values for each plate. For *S. griseus*, since a "zone" was not really visible around the discs, we resorted to counting the number of colonies present around each filter paper disc (colonies are visible in the image above). We then conducted an ANOVA test between PP fluid discs and DW discs on each plate to determine the statistical significance of our data.

Raw Measurements Taken From Zone of Inhibition Plates

Bacteria	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6
***E. coli* (PP fluid discs)**	1.371 cm	1.323 cm	1.452 cm	1.371 cm	1.419 cm	1.419 cm
***E. coli* (DW discs)**	0.065 cm	0.063 cm	0.087 cm	0.085 cm	0.138 cm	0.112 cm
***S. epidermidis* (PP fluid discs)**	1.157 cm	0.857 cm	0.857 cm	1.06 cm	1.151 cm	1.031 cm
***S. epidermidis* (DW discs)**	0.068 cm	0.131 cm	0.104 cm	0.057 cm	0.091 cm	0.055 cm

***S. griseus* (PP fluid discs)**	0	0	N/A	N/A	N/A	N/A
***S. griseus* (DW discs)**	3	8	N/A	N/A	N/A	N/A



ANOVA Test for Significant *E. coli* Data

Result Details

Source	*SS*	*df*	*MS*	
Between-treatments	2.6199	1	2.6199	*F* = 275.01114
Within-treatments	0.0953	10	0.0095	
Total	2.7151	11		

The *f*-ratio value is 275.01114. The *p*-value is < .00001. The result is significant at $p < .05$.

ANOVA Test for Significant *S. epidermidis* Data

Result Details				
Source	***SS***	***df***	***MS***	
Between-treatments	5.0765	1	5.0765	F = 3416.48767
Within-treatments	0.0149	10	0.0015	
Total	5.0914	11		

The *f*-ratio value is 3416.48767. The *p*-value is < .00001. The result is significant at $p < .05$.

ANOVA Test for Significant *S. griseus* Data

Result Details				
Source	***SS***	***df***	***MS***	
Between-treatments	30.25	1	30.25	F = 4.84
Within-treatments	12.5	2	6.25	
Total	42.75	3		

The *f*-ratio value is 4.84. The *p*-value is .158809. The result is *not* significant at $p < .05$.

Discussion

In our first trial, quite surprisingly, the only regions containing bacterial density on all plates were the regions around filter paper discs soaked in pitcher plant fluid. This is shown in *Figure* 2. There is a very real possibility that this fluid that we collected in this trial was simply residue leftover from watering the plant (it was not fluid produced by the pitcher plant). The pitcher plant might have also been malnourished and unable to produce normal fluid (or any fluid at all). The bacteria in this contaminated fluid might exist because of this standing water

being virtually untouched over several days while sitting inside the pitchers. Our difficulties with producing pitcher plant fluid are discussed below.

One of the biggest problems we had with obtaining meaningful results was the collection of proper pitcher plant fluid. When we received our pitcher plants, they were in good condition (showing no signs of decaying or dead flesh), but all pitchers were dry and had no fluid in them. We resorted to only moderate watering of the plant, as several online guides and papers studying *Nepenthes* stated that less frequent watering was best. After the plant began to wither and die, we elected to change our watering routine and water the plant more frequently (once every 1-2 days), using a spray bottle not only to water the soil but also to spray moisture onto the plant's leaves. The plant was also placed in a greenhouse to create humid conditions in which it survives best. Since we began that procedure, the plant has not shown definitive improvement or deterioration in its condition. Fluid from that plant was harvested and used in an initial zone of inhibition study, but that data yielded unsatisfactory and inconclusive results, so the data from that initial study was thrown out. We ultimately decided to order a new *Nepenthes* plant and harvest fluid immediately upon receiving that new plant. Once we received it, we collected its fluid and used it immediately. With this fluid, we obtained results that were statistically significant, and those are the results that are discussed in this report.

Using fluid from the new pitcher plants we ordered, very large zones of inhibition around the filter paper discs were observed. In comparison, the discs that were soaked in distilled water showed no inhibition whatsoever. This is shown in *Figure* 3. Obviously, the pitcher plant fluid has some sort of antimicrobial effect, and this data further demonstrated that the fluid used in our first trial was indeed contaminated, or was simply not pitcher plant fluid at all. The only reasonable explanation for the inhibition is the antimicrobial enzymes present in the fluid. For *E. coli* and *S. epidermidis*, the ANOVA test performed on the collected data for those two types of bacteria concluded that our data was indeed statistically significant. The absence of bacteria within the zone of inhibition on those two plates was a clear indication that the pitcher plant fluid had some sort of antimicrobial effect. As Hatano and Hamada noted in their paper, three out of seven of the enzymes they found in their proteomic analysis presented antimicrobial effects. This could be the sole reason why the bacteria do not grow in a radius around the filter paper disc. Another possible explanation for the lack of growth might be the nature of the fluid itself. Buch and Rott et al. noted that the fluid of *Nepenthes* pitcher plants is unsuitable for microbial growth due to the presence of antimicrobial naphthoquinones (2013). They also noted that the fluid simply does not contain the nutrients necessary for growth (phosphate and inorganic nitrogen in particular), (Buch & Rott et al. 2013). Though, the latter cannot be confirmed or denied because the plates on which the bacteria were grown in this inquiry were nutrient-rich through the LB media used. For *S. griseus*, even though the ANOVA test performed on our collected data did not show statistical significance (our p-value was 0.15, greater than the cutoff of 0.05), the qualitative data still shows some sort of inhibition of growth of *S. griseus*. Though, the absence of colonies around the filter paper discs soaked in PP fluid on this plate might be due to chance

alone. The colonies that grew around the distilled water discs might just have "gotten lucky." The colonies on those two discs might have just grown by chance.

Our data clearly demonstrated that pitcher plant fluid could be used as an antimicrobial agent against a wide variety of infective pathogens. The statistical significance of our data further reinforced the credibility of the antimicrobial effect of *Nepenthes* pitcher plant fluid. Our findings are meaningful for several reasons. First, the pitcher plant fluid could be used as a new disinfectant – one that is safe, natural/organic, and is not made of any toxic or harmful chemicals. This would be very appealing to many people, especially those who prefer organic and "all natural" products over synthetic ones. Further purification of the pitcher plant fluid might allow us to concentrate the antimicrobial enzymes/chemicals, further increasing the strength of the fluid. Second, this could mean a new antibiotic. This antibiotic is unique and resilient because it is made of an enzyme, not a small chemical compound. This means that it would be much harder for infectious bacteria to develop resistance to these large enzymes, making this new antibiotic very effective with little future worry about resistance.

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